PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Zuker *et al.*

Application No.: 09/361,652

Filed: July 27, 1999

For: **NUCLEIC ACIDS ENCODING A G-PROTEIN COUPLED RECEPTOR INVOLVED IN SENSORY TRANSDUCTION**

Examiner: Michael Brannock

Art Unit: 1646

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. CHARLES ZUKER

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Charles Zuker, Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I received my Ph.D. from Massachusetts Institute of Technology. I am currently a Professor and Investigator, Howard Hughes Medical Institute, Departments of Biology and Neurosciences, School of Medicine, University of California at San Diego. I have been in this position since 1986. See resume, Exhibit A.

4. The above-referenced patent application claims isolated nucleic acids encoding GPCR-B3, also known as T1R1, a taste bud specific G protein coupled receptor involved in taste transduction.

5. I am an inventor of the above-referenced patent application. I have read and am familiar with the contents of the patent application. In addition, I have read the Office Action, mailed August 12, 2001, received in the present case. It is my understanding that the Examiner believes that this invention is supported by neither a specific, substantial, and credible asserted utility nor a well established utility as required by the United States Patent Laws.

6. This declaration is provided to demonstrate that, at the time the application was filed, one of skill in the art would recognize the utility of the present invention and would appreciate its real world context.

7. The present application discloses that the claimed nucleic acid, a full length cDNA, encodes a G protein coupled receptor ("GPCR") that is specifically expressed in taste buds of the tongue, and provides data demonstrating that the claimed protein is a functional G-protein coupled receptor. The present invention is therefore useful, e.g., for screening for taste modulators of a taste bud cell specific GPCR, for the identification of GPCR-B3 taste ligands, and as a specific marker for specialized taste bud cells of the tongue.

8. As described in the present specification, full length cDNAs that encode a taste cell-specific nucleic acids were cloned. Sequence analysis of the GPCR-B3 clone showed that it had the structure of a G-protein coupled receptor, with an extracellular domain, seven transmembrane domains, and a cytoplasmic domain (*see, e.g.*, Example I, page 56-57). Subsequently, protein expression patterns were determined for GPCR-B3 using *in situ* analysis (*see, e.g.*, Example II, page 58, and Figure 3). Figure 3 shows that the claimed nucleic acids express proteins that are specifically expressed in taste buds of the tongue.

9. Furthermore, the specification provides experimental data demonstrating that GPCR-B3 is a functional G-protein coupled receptor. Figure 4 shows the structure of a chimeric protein, comprising an extracellular domain of a murine MGluR1 receptor fused to the seven transmembrane domains and cytoplasmic domains of GPCR-B3. This chimeric GPCR construct was transfected into HEK cells, which were then stimulated with glutamate, the MGluR1 ligand. The HEK cells demonstrated an increase in intracellular calcium in response to the ligand, indicating that the chimeric GPCR couples to a promiscuous G protein and triggers calcium responses that are detectable using the indicator fura-2. The presently claimed GPCR-B3 nucleic acids therefore encode a G protein coupled receptor that is specifically expressed in fungiform and foliate cells of the tongue, which are taste bud cells, as described in the specification.

10. It would be apparent to anyone of skill in the art that GPCR-B3 is an excellent target for candidate compounds that modulate taste transduction. This use is not merely a "starting point for further research and investigation," but a direct assay for taste ligands and modulators of taste signal transduction. Furthermore, the claimed nucleic acids are specifically expressed in a unique subset of tongue cells, and the encoded proteins localize to the taste pore- the subcellular location for taste receptors. As such, they have specific and substantial utility as markers for specialized taste cells of the tongue. Such markers are useful for the generation of taste topographic maps the

elucidate the relationship between taste bud cells of the tongue and taste sensory neurons leading to taste centers in the brain. Applicants have therefore provided a nucleic acid that encodes a protein with known signaling activity and specific expression in a specialized sub-set of cells.

11. In view of the foregoing, it is my scientific opinion that one of skill in the art, at the time the application was filed, would immediately recognize the real world utility of the nucleic acids of this invention. Therefore, this invention is supported by a specific, substantial, and credible utility.

Date: 9/10/02 By: _____

Charles Zuker, Ph.D.

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EDUCATION

INSTITUTION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Universidad Catolica de Valparaiso; Chile	B.Sc., Honors	1977	Biology
Massachusetts Inst. of Technology; Boston	Ph.D.	1983	Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE

1993 - present	Professor and Investigator; Howard Hughes Medical Institute Departments of Biology and Neurosciences, School of Medicine University of California, San Diego
1989 - 1992	Associate Professor and Associate Investigator Howard Hughes Medical Institute, UCSD
1986 - 1989	Assistant Professor; Department of Biology, UCSD
1983 - 1986	Postdoctoral Fellow; Department of Biochemistry; University of California, Berkeley
1977 - 1983	Graduate Student; Department of Biology; Massachusetts Institute of Technology

Honors and Keynote Lectures (selected)

Whitaker Health Sciences Fund Fellow, Massachusetts Inst. of Technology, 1979-1980
Whitaker Health Sciences Fund Fellow, Massachusetts Inst. of Technology, 1981-1982
European Molecular Biology Organization Fellow, 1983
Jane Coffin Childs Memorial Fund for Medical Research Fellow, 1984-1986
McKnight Foundation Fund for Neuroscience Award, 1988-1991
Monsanto Speaker, St. Louis University, St Louis, MO, 1991
Broadhurst Foundation visiting lecturer, Cambridge, MA, 1991
Institute Speaker, Scripps Research Institute, La Jolla, CA, 1992
Keynote speaker, Stanford Neurosciences Program Retreat, Monterey, CA,1992
Pew Scholars Award, 1988-1992
Alfred P. Sloan Award in Neurosciences, 1988-1990
March of Dimes Basil O'Connor Award, 1989-1991
Merck Lecturer, UC Berkeley 1992
Institute speaker, Roche Institute of Molecular Biology, Nutley, NJ, 1993
Keynote Speaker, Pharmacological Sciences Program, Vanderbilt University, Nashville, TN, 1994
Keynote Speaker, Stanford Medical Scientist Training Program, Stanford University CA, 1994
Lecturer in the Life Sciences, Northwestern University Medical School, Chicago, IL 1994

Howard Hughes Medical Institute, Lecture series to Institute employees, Howard Hughes Medical Institute, Chevy Chase, MD, 1996
Keynote Speaker, FASEB Summer Conference on "The Biology and Chemistry of Vision", Keystone, CO, 1997
Keynote Speaker, U. Penn Graduate programs in Biochemistry, Molecular Biology and Pharmacology. Philadelphia, 1998
Cogan Award, Association for Research in Vision and Ophthalmology, 1998
University Lecturer, UT Southwestern Medical School, 1999
Alcon Award for outstanding contributions to vision research, 1999
American Academy of Arts and Sciences, 2000

Study Sections and Advisory Boards (selected):
Member, Scientific Advisory Board, Pew Latin American Scholars Program, 1990 - present
Mechanisms of Development, 1991-present
Neuron, 1995-present
Member, American Cancer Society Postdoctoral Research Selection Committee, 1995-1999
Member, Scientific Advisory Board, Schepens Research Institute, Harvard University, Cambridge, MA, 1995 - present
Member, Review Panel, Howard Hughes Medical Institute International Grants Program, 1996
Member, National Research Council/ National Academy of Sciences advisory committee for the US and HHMI program in Latin America, 1997-
National Advisory Committee of The Pew Scholars Program in the Biomedical Sciences, 1997-
Member, NIH Visual Sciences C study section, Bethesda, MD, 1997-2000
Member, NIDCD Strategic Planning committee 1999-
Damon Runyon-Walter Winchell Cancer Fund Scientific Advisory Committee, 1999-
Current Biology, 2000-
Steering Committee, Alliance for Cellular Signaling, 2000-
Advisory board, Pew program in Science and Society, 2001-
Advisory board, NIH-wide initiative on mouse mutagenesis, 2001-

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X. EVIDENCE APPENDIX

2. Nelson *et al.* reference

An amino-acid taste receptor

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The sense of taste provides animals with valuable information about the nature and quality of food. Mammals can recognize and respond to a diverse repertoire of chemical entities, including sugars, salts, acids and a wide range of toxic substances[1]. Several amino acids taste sweet or delicious (umami) to humans, and are attractive to rodents and other animals[2]. This is noteworthy because L-amino acids function as the building blocks of proteins, as biosynthetic precursors of many biologically relevant small molecules, and as metabolic fuel. Thus, having a taste pathway dedicated to their detection probably had significant evolutionary implications. Here we identify and characterize a mammalian amino-acid taste receptor. This receptor, T1R1+3, is a heteromer of the taste-specific T1R1 and T1R3 G-protein-coupled receptors. We demonstrate that T1R1 and T1R3 combine to function as a broadly tuned L-amino-acid sensor responding to most of the 20 standard amino acids, but not to their D-enantiomers or other compounds. We also show that sequence differences in T1R receptors within and between species (human and mouse) can significantly influence the selectivity and specificity of taste responses.

T1Rs and T2Rs are two families of G-protein-coupled receptors (GPCRs) selectively expressed in subsets of taste receptor cells[3–11]. T2Rs are involved in bitter taste detection[4,5], and T1R2 and T1R3 combine to function as a sweet taste receptor[7]. To identify taste receptors involved in amino-acid detection, we used an expression screening strategy similar to that used in the characterization of bitter and sweet taste receptors. Candidate receptors were expressed in human embryonic kidney (HEK) cells containing the $G\alpha_{16}$–$G\alpha_z$ and $G\alpha_{15}$ promiscuous G proteins[12,13], and assayed for stimulus-evoked changes in intracellular calcium. In this system, receptor activation leads to activation of phospholipase Cβ (PLC-β) and release of calcium from internal stores, which can be monitored at the single-cell level using calcium-indicator dyes[5,7,14].

Because T1R taste receptors are distantly related to GPCRs that recognize the amino acids glutamate[15] (metabotropic glutamate receptors, mGluRs), GABA[16] (γ-aminobutyric acid; GABA-B receptors) and arginine[17] (the R5-24 receptor), we began by testing members of the T1R family. Patterns of T1R expression define at least three distinct cell types: cells co-expressing T1R2 and T1R3 (T1R2+3, a sweet receptor), cells co-expressing T1R1 and T1R3 (T1R1+3) and cells expressing T1R3 alone[7]. First, we assayed responses of the T1R2+3 sweet taste receptor to all 20 standard and various D-amino acids. Several D-amino acids that taste sweet to humans, and are attractive to mice, trigger robust activation of the T1R2+3 sweet taste receptor (Fig. 1a, b). However, none of the tested L-amino acids activate this receptor.

Mouse T1R1 and T1R3 were transfected alone or in combination and tested for stimulation by L-amino acids. Individual receptors showed no responses. In contrast, T1R1 and T1R3 combine to function as a broadly tuned L-amino-acid receptor, with most amino acids that are perceived as sweet (for example, alanine, glutamine, serine, threonine and glycine[2]) activating T1R1+3 (Fig. 1). The responses are strictly dependent on the combined presence of T1R1 and T1R3, and are highly selective for L-amino acids; D-amino acids and other natural and artificial sweeteners did not activate the T1R1+3 receptor combination. These results substantiate T1R1+3 as a receptor for L-amino acids, and provide a striking example of heteromeric GPCR receptors radically altering their selectivity by a combinatorial arrangement of subunits.

If T1R1+3 functions as a major L-amino acid taste sensor *in vivo*, we might expect its cell-based behaviour to recapitulate some of the physiological properties of the *in vivo* receptor. Nerve recordings in rats have shown that taste responses to L-amino acids are considerably potentiated by purine nucleotides such as inosine monophosphate (IMP)[18]. To assay the effect of IMP, HEK cells expressing the T1R1+3 receptor combination were stimulated with amino acids in the presence or absence of IMP. Indeed, T1R1+3 responses to nearly all L-amino acids were dramatically enhanced by low doses of IMP (Figs 1 and 2a); this effect increased over a range of 0.1–10 mM (Fig. 2b). However, IMP alone elicited no response, even at the highest concentration tested in our assays, and it had no effect on responses mediated by T1R2+3 (either to sweeteners or to L- and D-amino acids; data not shown).

T1R1+3 is prominently expressed in fungiform taste buds[7], which are innervated by chorda tympani fibres. Therefore, we stimulated mouse fungiform papillae at the front of the tongue with various amino acids in the presence or absence of IMP, and recorded tastant-induced spikes from the chorda tympani nerve. As expected, nerve responses to L-amino acids were significantly enhanced by IMP[18] (Fig. 3). However, IMP had no significant effect on responses to D-amino acids or to non-amino-acid stimuli.

Genetic studies of sweet tasting have identified a single principal locus in mice influencing responses to several sweet substances (the *Sac* locus[19,20]). Sac 'taster' mice are about fivefold more sensitive to sucrose, saccharin and other sweeteners than Sac non-tasters. *Sac* codes for T1R3[7–11,21]. There are two amino-acid differences that define taster and non-taster alleles[7,9,10]. One of these changes, I60T, introduces a potential glycosylation site that was proposed to eliminate receptor function by preventing receptor dimerization[10]. This poses a conundrum because responses to L-amino acids are not influenced by the *Sac* locus[7,22] (and data not shown). Thus, if T1R3 functions as the common partner of the sweet and amino-acid receptors, we reasoned that the T1R3 non-taster allele must selectively affect the T1R2+3 combination.

We examined the effect of the *Sac* non-taster allele on T1R1 and T1R2 using biochemical and functional assays. First, we investigated receptor heteromerization by co-immunoprecipitating differentially tagged T1R receptors. In essence, HEK cells were co-transfected with taster and non-taster alleles of T1R3 and either haemagglutinin (HA)-tagged T1R1 or T1R2. Receptor complexes were then immunoprecipitated with anti-HA antibodies, and the association with T1R3 assayed with anti-T1R3 antibodies. Other results demonstrated that the non-taster form of T1R3, much like its taster counterpart, assembles into heteromeric receptors with T1R1 and T1R2 (Fig. 4a). This argues against the possibility that the sweet taste deficits of Sac non-taster animals result from failure to assemble heteromeric receptors. Second, we examined the functional responses of T1R2+3 (sweet) and T1R1+3 (amino acid) receptors carrying either the taster or non-taster allele of T1R3. The taster and non-taster alleles of T1R3 generate functionally similar receptors when combined with T1R1, but the non-taster form displays significantly impaired responses when combined with T1R2 (Fig. 4b). Thus, responses to L-amino acids are not affected by the *Sac* locus in mice because *Sac* selectively affects the T1R2+3 receptor combination.

The finding that polymorphism in one of the T1R receptor

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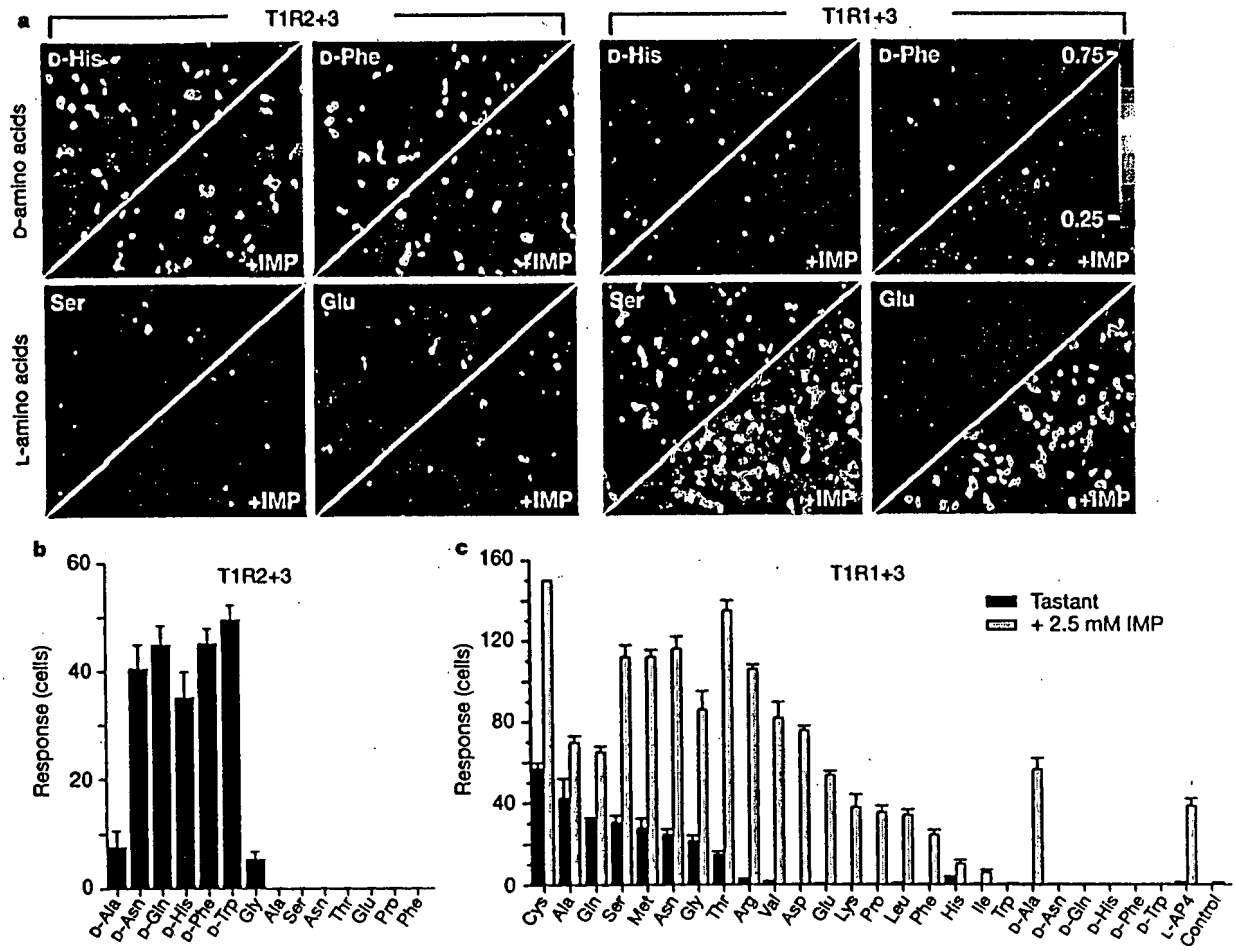

Figure 1 T1R receptor combinations respond differentially to L- and D-amino acids. **a**, HEK-293 cells co-expressing promiscuous G proteins and heteromeric mouse T1R2+3 or T1R1+3 receptors were stimulated with L- and D-amino acids. The T1R2+3 sweet taste receptor is activated by sweet-tasting D-amino acids but not by L-amino acids (left). In contrast, T1R1+3 is activated by L-amino acids and responses are potentiated by IMP (right). Amino acids were 50 mM and IMP was 2.5 mM; the colour scale indicates the F_{340}/F_{380} ratio (see Methods). **b**, **c**, Quantification of amino-acid responses for T1R2+3 (**b**) and T1R1+3 (**c**). Amino acids were 50 mM, and IMP and L-AP4 were 2.5 mM; control refers to 2.5 mM IMP alone. Each column represents the mean ± s.e.m. of at least ten independent determinations. IMP had no effect on T1R2+3 (data not shown). D-Amino acids (with the exception of D-Ala in the presence of IMP) and natural or artificial sweeteners did not activate T1R1+3. Trp elicited no responses and Tyr was not assayed because it is insoluble at high concentration. Note that the achiral amino acid Gly activates both receptor complexes. All calcium measurements and quantifications were performed as described in the Methods and ref. 7.

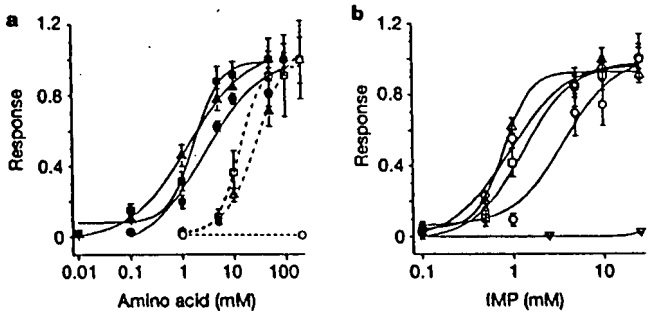

Figure 2 Dose response of T1R1+3 to L-amino acids and IMP. **a**, Dashed lines with open symbols represent dose responses of T1R1+3 with L-amino acids (squares, Ala; circles, Glu; triangles, Ser). The presence of 2.5 mM IMP (solid lines with filled symbols) shifts the responses by at least one order of magnitude to the left. Equivalent results were obtained with most L-amino acids (see also Fig. 1b). **b**, IMP potentiates responses of T1R1+3. Shown are dose responses for Ala (2 mM, squares), Glu (4 mM, circles), Ser (2 mM, triangles), Gly (4 mM, diamonds) and IMP (inverted triangles). Responses were normalized to the mean response at the highest concentration. Each point represents the mean ± s.e.m of at least ten assays.

subunits differentially affects receptor function suggests that other sequence variations in the amino-acid and sweet receptors may significantly influence tastant sensitivity or selectivity. For example, humans can taste a number of artificial sweeteners that rodents cannot (for example, aspartame, cyclamate and various sweet proteins[23]). Rodent and human T1Rs are only about 70% identical[7]. Therefore, we generated heteromeric receptors consisting of human and rodent T1R subunits and assayed for activation by amino acids and artificial sweeteners. Indeed, the presence of human T1R1 or T1R2 greatly altered the sensitivity (Fig. 4c) and the specificity (Fig. 4d) of the amino acid sweet receptors. Cells expressing human T1R1 are more than an order of magnitude more sensitive to glutamate than to other amino acids, and cells expressing human T1R2 robustly respond to aspartame, cyclamate and intensely sweet proteins (Fig. 4d and data not shown). Thus, the nature of the unique partner determines whether the receptor complex will function as a sweet receptor or as an amino-acid receptor, and sequence differences in T1Rs between or within species (for example, polymorphisms in *Sac*) can greatly influence taste perception.

In humans, monosodium L-glutamate (MSG) elicits a unique

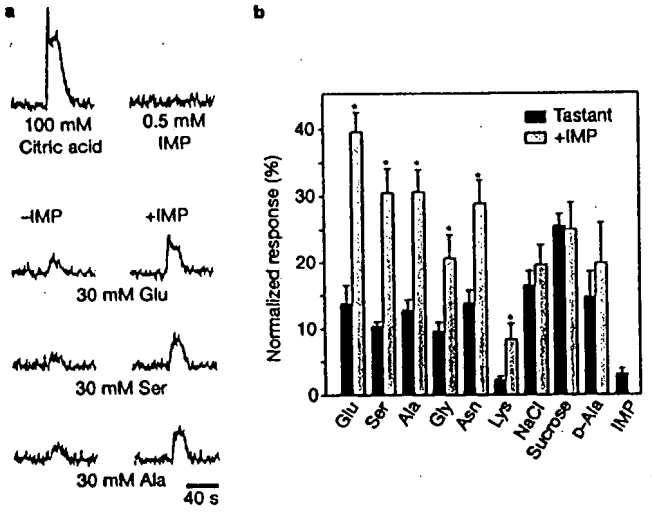

Figure 3 IMP stimulates responses of the chorda tympani nerve to amino acids in mice. **a**, Integrated neural responses of C57BL/6 mice to Glu, Ser and Ala (30 mM each) were recorded with and without 0.5 mM IMP. The responses to 100 mM citric acid and 0.5 mM IMP alone are shown in the upper traces. Equivalent results were obtained for most L-amino acids. **b**, Integrated neural responses, such as those shown in **a**, were normalized to the responses of 100 mM citric acid. Black bars, tastant alone; grey bars, tastant + 0.5 mM IMP. The values are means ± s.e.m. ($n = 5$). Sucrose was used at 100 mM and all other tastants at 30 mM. Asterisks indicate statistically significant differences ($P < 0.05$).

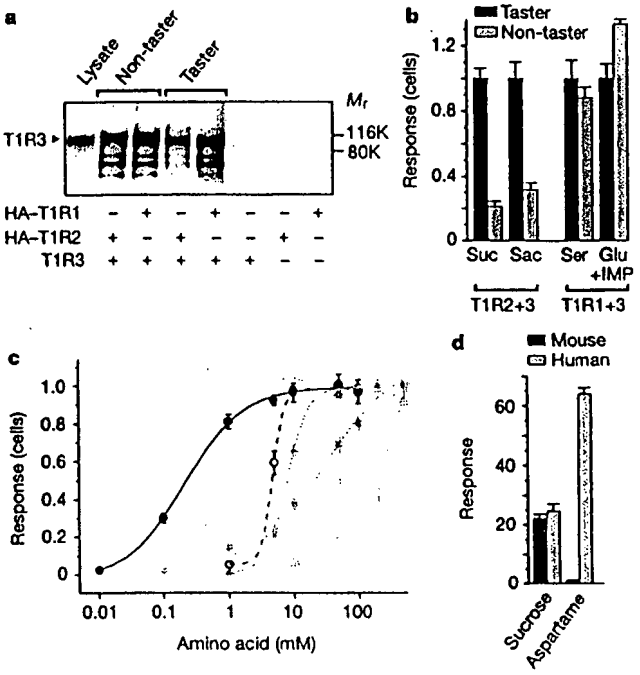

Figure 4 Polymorphic differences in T1Rs influence receptor function. **a**, Immunoprecipitation and western blot analyses shows that *Sac* non-taster and taster alleles of T1R3 form heteromeric complexes with T1R1 and T1R2. Cells were transfected with combinations of T1Rs as indicated. All extracts were immunoprecipitated with anti-HA antibodies, and the resulting protein complexes probed with anti-T1R3 antibodies. M_r, relative molecular mass, in thousands (K). **b–d**, Results of cell-based calcium imaging assays. **b**, The *Sac* allele selectively affects the T1R2+3 heteromeric receptor. Responses were normalized to the mean responses obtained with the taster allele (black bars). The responses of T1R2+3 to sweet compounds are significantly reduced when the non-taster T1R3 allele is used, but responses of T1R1+3 to amino acids are unaffected, even in the presence of IMP. **c**, Human T1R1 influences sensitivity to monosodium L-glutamate. Low-concentration MSG robustly activates receptors containing human T1R1 (open circles), and IMP potentiates the response (filled circles). Also shown for comparison are dose responses for Ala (squares) and Ser (triangles). For each series, responses were normalized to the mean response at the highest concentration. **d**, Mouse T1R2+3 (black bars) responds to sucrose and other natural and artificial sweeteners, but not aspartame. However, substituting human T1R2 for mouse T1R2 (grey bars) in the rodent T1R2+3 receptor imparts aspartame sensitivity.

savoury taste sensation called umami[24,25]. Hallmarks of the umami taste are its potentiation by purine nucleotides, and activation by the mGluR-agonist L-AP4 (ref. 25). A mGluR4 splice variant has recently been isolated as a candidate umami receptor[26]. An important question is whether T1R1+3 is umami receptor. Our results demonstrate that T1R1 and T1R3 combine to function as a broadly tuned amino-acid receptor. Notably, T1R1+3 responses to L-AP4 (Fig. 1), MSG and other amino acids are greatly potentiated by purine nucleotides. Thus, we propose that T1R1+3 is a constituent of the umami response. Future studies should help define whether T1R1+3 is the principal, or an additional, umami receptor. An interesting paradox that emerged from this work is the relationship between receptor activity and taste perception. For example, T1R1+3 responds to most L-amino acids, but not all amino acids taste the same: some are attractive to mice and sweet to humans, whereas others are neutral; some are even perceived as bitter and are aversive to animals[2]. Similarly, very few amino acids elicit the taste of umami. The identification of bitter, sweet, and now an amino-acid taste receptor provide a powerful platform to help decode the interplay between the various taste modalities, and the link between events at the periphery (taste receptor cells) and the central nervous system (perception and behaviour). □

Methods

Heterologous expression and calcium imaging

Cells were grown, maintained and transfected exactly as described earlier[7]. Transfection efficiencies were estimated by co-transfection with a green fluorescent protein (GFP) reporter plasmid and were typically >70%. FURA-2 acetomethyl ester was used to measure intracellular calcium concentration ($[Ca^{2+}]_i$), and assay conditions were identical to those previously described[7]. Responses were measured for 60 s and the fluorescence ratio at wavelengths of 340 and 380 nm (F_{340}/F_{380}) was used to measure $[Ca^{2+}]_i$. For data analysis, response refers to the number of cells responding in a field of about 300 transfected cells. Cells were counted as responders if F_{340}/F_{380} increased above 0.27 after addition of tastant. In general, >90% of the responding cells had $F_{340}/F_{380} > 0.35$. Dose-response functions were fitted using the logistical equation. Studies involving taster and non-taster alleles of T1R3 used contructs of complementary DNA coding for T1R3 from C57BL/6 and 129/Sv mice, respectively[7–11,21].

Immunoprecipitation

Antibodies against T1R3 were generated using a peptide corresponding to residues 824–845 of the mouse receptor. PEAK^{rapid} cells (Edge Biosciences) were transfected with HA–T1R1, HA–T1R2 and T1R3 in various combinations and were gathered and disrupted in buffer containing 50 mM Tris-HCl at pH 7.5, 300 mM NaCl, 1% NP-40, 0.5% w/v sodium deoxycholate, and protease inhibitors (Roche). Lysates were incubated overnight at 4 °C with mouse monoclonal anti-HA antibody (Santa Cruz) and immune complexes were collected with protein AG–agarose beads. Samples were fractionated by SDS–PAGE, transferred to nitrocellulose membrane and probed with anti-T1R3 antibody. As a control for the specificity of the interactions, we have shown that artificially mixing extracts from cells expressing tagged T1R1 or T1R2 with extracts from cells expressing T1R3 does not produce complexes. Similarly, co-transfection of a Rho-tagged mGluR1 receptor[15] did not produce T1R–GluR1 complexes.

Nerve recording

Lingual stimulation and recording procedures were performed as previously described[27]. Neural signals were amplified (2,000×) with a Grass P511 AC amplifier (Astro-Med), digitized with a Digidata 1200B A/D convertor (Axon Instruments), and integrated (r.m.s. voltage) with a time constant of 0.5 s. Taste stimuli were presented at a constant flow rate of 4 ml min^{-1} for 20-s intervals interspersed by 2-min rinses between presentations. All data analyses used the integrated response over a 25-s period immediately after the application of the stimulus. Each experimental series consisted of the application of six tastants bracketed by presentations of 0.1 M citric acid to ensure the stability of the recording. The mean response to 0.1 M citric acid was used to normalize responses to each experimental series.

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Competing interests statement

The authors declare that they have no competing financial interests.

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